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mTOR activation induces tumor suppressors that inhibit leukemogenesis and deplete hematopoietic stem cells after *Pten* deletion

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SUMMARY

Pten deficiency depletes hematopoietic stem cells (HSCs) but expands leukemia-initiating cells and the mTOR inhibitor, rapamycin, blocks these effects. Understanding the opposite effects of mTOR activation on HSCs versus leukemia-initiating cells could improve anti-leukemia therapies. We found that the depletion of *Pten*-deficient HSCs was not caused by oxidative stress and could not be blocked by N-acetyl-cysteine. Instead, *Pten* deletion induced, and rapamycin attenuated, the expression of $p16^{Ink4a}$ and p53 in HSCs, and $p19^{Arf}$ and p53 in other hematopoietic cells. p53 suppressed leukemogenesis and promoted HSC depletion after *Pten* deletion. $p16^{Ink4a}$ also promoted HSC depletion but had a limited role suppressing leukemogenesis. $p19^{Arf}$ strongly suppressed leukemogenesis but did not deplete HSCs. Secondary mutations attenuated this tumor suppressor response in some leukemias that arose after *Pten* deletion. mTOR activation therefore depletes HSCs by a tumor suppressor response that is attenuated by secondary mutations in leukemogenic clones.

Keywords

Pten; hematopoietic stem cell; leukemia-initiating cell; mTOR; Ink4a/Arf; p53

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AUTHOR CONTRIBUTIONS

J.Y.L. and D.N. performed all experiments and participated in the design and interpretation of experiments. O.H.Y. initiated the project and generated the compound mutant mice. M.S.L. analyzed mouse pathology with help from J.Y.L. Z.T. and D.G.G. participated in the conception and interpretation of experiments. S.J.M. participated in the design and interpretation of *Pten* experiments and wrote the paper with J.Y.L and D.N.

INTRODUCTION

Phosphatidylinositol 3-kinase (PI-3kinase) pathway signaling promotes cell growth, proliferation, and survival by several mechanisms (Wullschleger et al., 2006; Yuan and Cantley, 2008) (see Fig. S1 for a schematic). Receptor tyrosine kinases and other signaling pathways activate PI-3kinase, which generates phosphatidylinositol-3,4,5-trisphosphate (PIP3) (Yuan and Cantley, 2008). PIP3 activates Akt, which promotes cell growth, proliferation, and survival by phosphorylating diverse substrates (Manning and Cantley, 2007), including the Tuberous Sclerosis Complex (TSC) (Inoki et al., 2002). Phosphorylation by Akt negatively regulates TSC, leading to the activation of the mammalian target of rapamycin (mTOR) kinase (Inoki et al., 2002). mTOR functions in two distinct complexes, mTORC1, which is directly inhibited by rapamycin, and mTORC2, which can be indirectly inhibited by rapamycin (Guertin and Sabatini, 2007; Sarbassov et al., 2006). mTORC1 promotes cell growth and proliferation by activating S6 kinase and inactivating 4EBP1, promoting protein synthesis (Inoki et al., 2002). mTORC2 regulates Akt activation (Guertin and Sabatini, 2007). PI-3kinase signaling is attenuated by Pten, which dephosphorylates PIP3 (Maehama and Dixon, 1998), reducing the activation of Akt, mTORC1, and S6 kinase. As a result, *Pten* deficiency increases the growth, proliferation, and survival of many cells (Sun et al., 1999) and *Pten* is commonly deleted in diverse cancers (Di Cristofano and Pandolfi, 2000).

PI-3kinase pathway signaling has divergent effects on stem cells. Conditional deletion of *Pten* from embryonic stem cells and neural stem cells increases cell cycle entry and self-renewal (Gregorian et al., 2009; Groszer et al., 2006; Groszer et al., 2001; Sun et al., 1999). In contrast, *Pten* deletion from adult HSCs increases cell cycle entry but this leads to rapid HSC depletion (Yilmaz et al., 2006; Zhang et al., 2006). We showed that this depletion was mediated by mTOR activation as rapamycin blocked the depletion of *Pten*-deficient HSCs (Yilmaz et al., 2006). Subsequent studies of *Tsc1*-deficient HSCs confirmed that increased PI-3kinase pathway signaling can drive HSCs into cycle but that this leads to mTOR-mediated HSC depletion (Chen et al., 2008; Gan et al., 2008). *Pml* deletion also increased HSC cycling and led to mTOR-mediated HSC depletion (Ito et al., 2008). mTOR is thus a critical modulator of stem cell maintenance, raising the question of how mTOR activation leads to stem cell depletion.

While *Pten* deletion leads to the depletion of normal HSCs, this leads to the generation and expansion of leukemia-initiating cells (Yilmaz et al., 2006). This makes it possible to deplete leukemia-initiating cells while rescuing normal HSC function using rapamycin in mice that are deficient for *Pten* or *Pml* (Ito et al., 2008; Yilmaz et al., 2006). A sophisticated understanding of PI-3kinase pathway signaling can therefore lead to therapies that eliminate cancer-initiating cells with limited toxicity to normal stem cells.

One mechanism by which *Pten* deletion and PI-3kinase pathway activation could deplete stem cells involves a tumor suppressor response. Sustained oncogenic signals can induce tumor suppressors that cause cellular senescence (Lin et al., 1998; Serrano et al., 1997). Conditional inactivation of *Pten* in prostate leads to the induction of p53-mediated senescence, impeding the development of prostate cancer (Chen et al., 2005). These studies raise the question of whether a similar tumor suppressor response occurs after *Pten* deletion in stem cells, and whether this suppresses leukemogenesis or depletes HSCs.

Another mechanism by which *Pten* deletion could deplete HSCs involves the inactivation of FoxO family transcription factors. When localized to the nucleus, FoxO transcription factors promote the expression of enzymes that eliminate reactive oxygen species (ROS). However, activated Akt phosphorylates FoxO proteins, leading to their retention in the cytoplasm

(Biggs et al., 1999; Brunet et al., 1999) and increasing ROS levels. HSCs are particularly sensitive to the toxic effects of ROS (Ito et al., 2004; Ito et al., 2006). Deletion of *FoxO1/3/4*, or *FoxO3a* alone, from adult HSCs leads to increased ROS levels and HSC depletion that can be at least partially rescued by the antioxidant N-Acetyl-cysteine (NAC) (Miyamoto et al., 2007; Tothova et al., 2007; Yalcin et al., 2008). The depletion of *Tsc1*-deficient HSCs is also partly rescued by NAC treatment (Chen et al., 2008). On the other hand, constitutively active Akt did not increase ROS levels in HSCs and the depletion of these HSCs could not be rescued by NAC treatment (Kharas et al., 2010). These results raise the question of whether *Pten* deficiency and *FoxO* deficiency deplete HSCs by similar or different mechanisms.

We found that *Pten* deficiency and *FoxO1/3/4* deficiency lead to HSC depletion by different mechanisms. We were unable to detect FoxO3a inactivation or a significant increase in ROS levels in HSCs after *Pten* deletion. We were not able to rescue the depletion of *Pten*-deficient HSCs by treating with NAC. Instead, *Pten* deletion induced the expression of $p16^{Ink4a}$ and p53 in HSCs, and $p19^{Arf}$ and p53 in other hematopoietic cells. Analysis of compound mutant mice revealed that deficiency for $p19^{Arf}$, or $p16^{Ink4a}/p19^{Arf}$, or *p53* (but not $p16^{Ink4a}$) accelerated leukemogenesis after *Pten* deletion. Moreover, deficiency for $p16^{Ink4a}$, or $p16^{Ink4a}/p19^{Arf}$, or *p53* (but not $p19^{Arf}$) autonomously prolonged the reconstituting capacity of HSCs after *Pten* deletion. Our results demonstrate there are multiple mechanisms by which increased PI-3kinase pathway signaling can deplete HSCs, including an mTOR-mediated tumor suppressor response that occurs in HSCs after *Pten* deletion and that is attenuated by secondary mutations in leukemogenic clones.

RESULTS

Akt activation after *Pten* deletion activates mTORC1 but does not inactivate FoxO3a

We conditionally deleted *Pten* from HSCs and other hematopoietic cells in young adult $Pten^{fl/fl}$;*Mx-1-Cre*$^{+}$ mice and $Pten^{+/fl}$;*Mx-1-Cre*$^{+}$ littermate controls by administering seven doses of polyinosine-polycytidine (pIpC) over 14 days (Yilmaz et al., 2006). *Pten*-deleted mice and littermate controls were then treated for a week with daily injections of rapamycin or vehicle. Rapamycin treatment and *Pten* deletion both increased Akt activation in bone marrow cells and in HSCs based on Western blots performed in c-kit^{+}Flk-2^{-}Lin^{-}Sca-1^{+}CD48^{-} cells, a population highly enriched for HSCs (Christensen and Weissman, 2001; Kiel et al., 2005) (Fig. 1A, B). The increase in Akt phosphorylation after rapamycin treatment is consistent with the attenuation of a negative feedback loop from S6 kinase to IRS-1 as a result of reduced mTORC1 activation (Harrington et al., 2004). Rapamycin does not, therefore, rescue the reconstituting activity of *Pten*-deficient HSCs by normalizing Akt activation.

Pten deletion did not appear to deplete HSCs by negatively regulating FoxO expression. By western blot neither *Pten* deletion nor rapamycin treatment appeared to significantly affect total FoxO3a levels in c-kit^{+}Flk-2^{-}Lin^{-}Sca-1^{+}CD48^{-} HSCs (Fig. 1B), or phospho-FoxO3a levels, total FoxO1 levels, or phospho-H2AX levels in c-kit^{+}Lin^{-}Sca-1^{+} cells (Fig. S2). In control experiments, when CD150^{+}CD48^{-}CD41^{-}Lin^{-}c-kit^{+}Sca-1^{+} HSCs (Kiel et al., 2005) were sorted from control and *Pten*-deleted mice and stimulated with SCF and TPO in culture for 24 hours they showed an overall decrease in FoxO3a staining as well as decreased nuclear staining and increased cytoplasmic staining (Fig. S3). In contrast, when HSCs were stained immediately after isolation from mice, we detected no change in FoxO3a expression or subcellular localization after *Pten* deletion (Fig. 1F–H): FoxO3a continued to localize to the nucleus.

mTORC1 activity was clearly elevated after *Pten* deletion based on increased phospho-S6 levels in freshly isolated bone marrow cells and HSCs (Fig. 1A, 1B, 1D, 1E, 1H). This increase in phospho-S6 levels was attenuated by rapamycin treatment (Fig. 1A, 1B). These changes were further confirmed by increased phospho-4EBP1 levels in bone marrow cells after *Pten* deletion (Fig. 1A). This increase in phospho-4EBP1 levels was also attenuated by rapamycin treatment (Fig. 1A). Thus, *Pten* deletion increased mTORC1 and S6 kinase activation in a manner that was reversed by rapamycin, suggesting that the changes that lead to HSC depletion after *Pten* deletion are mediated by this branch of the PI-3kinase pathway.

Pten deletion increases ROS levels in thymocytes but not in HSCs or bone marrow cells

We assessed the intracellular levels of ROS in $CD150^{+}CD48^{-}CD41^{-}Lin^{-}c\text{-}kit^{+}Sca\text{-}1^{+}$ HSCs, $CD150^{-}CD48^{-}CD41^{-}Lin^{-}c\text{-}kit^{+}Sca\text{-}1^{+}$ transiently reconstituting multipotent progenitors (MPPs) (Kiel et al., 2008), bone marrow cells, and thymocytes from *Pten*-deleted mice and littermate controls one week after finishing pIpC treatment. After *Pten* deletion, we detected a significant increase in ROS levels in thymocytes (Fig. 2C, 2E; 2.8-fold, $p<0.05$) but not in bone marrow cells, HSCs, or MPPs (Fig. 2B, E). Three weeks after finishing pIpC treatment we also did not detect an increase in ROS levels within unfractionated bone marrow cells, HSCs, or MPPs (Fig. 2D, F). Only mice showing no signs of neoplasms were used in these experiments.

The increase in ROS levels in the thymus after *Pten* deletion was attenuated by daily subcutaneous injection of the anti-oxidant NAC, but NAC did not affect ROS levels in bone marrow cells, HSCs, or MPPs (Fig. 2F). It is unclear why *Pten* deletion increased ROS levels in thymocytes but not in HSCs or WBM cells, but ROS regulation is known to differ among hematopoietic cells (Ito et al., 2004;Ito et al., 2006;Tothova et al., 2007). We were also unable to detect significant effects of *Pten* deletion on the expression of FoxO target genes (Tothova et al., 2007) or antioxidant response genes in bone marrow cells, HSCs, or MPPs (Fig. S4C–H).

NAC does not rescue major hematopoietic defects after *Pten* deletion

Consistent with the normal ROS levels in HSCs and most other hematopoietic cells, daily treatment with NAC for 3 weeks after pIpC treatment did not rescue the changes in spleen, thymus, or bone marrow cellularity after *Pten* deletion (Fig. S4A, B). NAC treatment also did not rescue the depletion of bone marrow HSCs (Fig. 2G, J). MPP frequency and number in the bone marrow and spleen were not significantly affected by *Pten* deletion or NAC treatment (Fig. 2H, I, K, L). The frequency and absolute number of HSCs and MPPs increased significantly in the spleen three weeks after pIpC treatment (Fig. 2I, L), consistent with the extramedullary hematopoiesis that arises after *Pten* deletion (Yilmaz et al., 2006). NAC attenuated the increase in splenic HSCs, though HSC frequency and number still increased significantly in the spleen (Fig. 2I, L). NAC also had little effect on other changes in hematopoiesis after *Pten* deletion (Fig. S5A–C). The inability of NAC to rescue hematopoietic defects after *Pten* deletion contrasts with the ability of rapamycin to do so (Yilmaz et al., 2006).

To test whether NAC rescues the function of *Pten*-deleted HSCs, we treated $Pten^{fl/fl}$;*Mx-1-Cre*$^{+}$ and $Pten^{+/fl}$;*Mx-1-Cre*$^{+}$ mice with 7 doses of pIpC over 14 days, then transplanted 10 donor $CD150^{+}CD48^{-}CD41^{-}Lin^{-}c\text{-}kit^{+}Sca\text{-}1^{+}$ HSCs along with a radioprotective dose of recipient bone marrow cells into irradiated recipients. Half of the recipients received daily subcutaneous injections of NAC, while the other half received daily saline injections, starting on the day of transplantation. In three independent experiments, all recipients of control HSCs, whether treated with NAC or vehicle, were long-term multilineage reconstituted by donor cells (Fig. 3A–E). All of the recipients of *Pten*-deleted HSCs showed

transient multilineage reconstitution, irrespective of whether they were treated with NAC (Fig. 3A–E). The inability of NAC to rescue *Pten* deficient HSC frequency or function suggests oxidative stress is unlikely to be the primary determinant of HSC depletion after *Pten* deletion.

To assess whether NAC treatment reduces leukemogenesis after *Pten* deletion, we transplanted 1×10^6 bone marrow cells from untreated *Pten*$^{fl/fl}$*;Mx-1-Cre*$^{+}$ mice or *Pten*$^{+/fl}$*;Mx-1-Cre*$^{+}$ controls along with 500,000 recipient bone marrow cells into irradiated recipients. Six weeks later, we treated the recipients with seven doses of pIpC over 14 days followed by daily NAC or saline injection. In two independent experiments, recipients of control cells all survived for 120 days and showed no signs of leukemogenesis (n=20, Fig. 3F). In contrast, all 20 recipients of *Pten*-deleted bone marrow died with myeloproliferative disease (MPD) and/or T-cell acute lymphoblastic leukemia (T-ALL) 33 to 112 days after pIpC treatment, irrespective of whether they were treated with NAC (Fig. 3F; Fig.. S5D–I). NAC treatment did not significantly affect the lifespan of mice after *Pten* deletion (Fig. 3F) in contrast to rapamycin, which prevents leukemogenesis and extends the lifespan of *Pten*-deleted mice (Yilmaz et al., 2006).

Pten deletion leads to a tumor suppressor response in HSCs

We tested whether *Pten* deletion induced a tumor suppressor response by assessing the expression of $p16^{Ink4a}$, $p19^{Arf}$, p53, and $p21^{Cip1}$ in splenocytes. We did not detect $p16^{Ink4a}$ protein expression in splenocytes with or without *Pten* deletion (Fig. 4A). We did detect very low levels of *p16*^{Ink4a} transcript in splenocytes, but transcript levels were not significantly affected by *Pten* deletion or rapamycin treatment (Fig. S6A). In contrast, we observed clear increases in $p19^{Arf}$, p53, and $p21^{Cip1}$ protein after *Pten* deletion (Fig. 4A). These increases were attenuated or eliminated by rapamycin, suggesting that the expression of these tumor suppressors was elevated as a consequence of mTOR activation (Fig. 4A). We confirmed significantly increased expression of *p19*^{Arf} (Fig. 4B) and *p21*^{Cip1} (Fig. S6B) by qPCR in *Pten*-deficient splenocytes, and that these increases were eliminated by rapamycin treatment. We observed no effect of *Pten* deletion or rapamycin treatment on *p53* transcript levels (Fig. S6C), consistent with the observation that p53 expression is mainly regulated post-transcriptionally (Olsson et al., 2007).

We hypothesized that this tumor suppressor response inhibited leukemogenesis but promoted the depletion of HSCs after *Pten* deletion, and that rapamycin rescued HSC depletion by attenuating the tumor suppressor response in HSCs. Since $p16^{Ink4a}$ and $p19^{Arf}$ are expressed at extraordinarily low levels in primary somatic cells and only limited amounts of protein are available from rare HSCs, we sorted 2×10^6 $Lin^{-}c\text{-}kit^{+}$ hematopoietic stem/progenitor cells, immunoprecipitated $p16^{Ink4a}$, $p19^{Arf}$, and p53, then performed Western blots to assess the levels of $p16^{Ink4a}$, $p19^{Arf}$, and p53. We observed $p16^{Ink4a}$ and p53 expression in the *Pten*-deficient $Lineage^{-}c\text{-}kit^{+}$ cells but not in wild-type cells or cells treated with rapamycin, but did not detect $p19^{Arf}$ expression in wild-type or *Pten*-deficient $Lin^{-}c\text{-}kit^{+}$ cells (Fig. 4C).

p16^{Ink4a} transcript levels also increased in $CD150^{+}CD48^{-}CD41^{-}Lin^{-}c\text{-}kit^{+}Sca\text{-}1^{+}$ HSCs after *Pten* deletion and this increase was attenuated by rapamycin treatment (Fig. 4D). We were unable to detect a clear increase in *p19*^{Arf} expression after *Pten* deletion (Fig. 4D). We also stained $CD150^{+}CD48^{-}CD41^{-}Lin^{-}c\text{-}kit^{+}Sca\text{-}1^{+}$ HSCs with anti-p53 antibody on slides to assess the level of p53 expression on a cell-by-cell basis. *Pten* deficient HSCs that had not been treated with rapamycin exhibited significantly increased p53 staining (Fig. 4E, F). Together, our results suggest that $p16^{Ink4a}$ and p53, but not $p19^{Arf}$, are induced in HSCs after *Pten* deletion.

Deficiency for $p19^{Arf}$ or p53, but not $p16^{Ink4a}$, accelerates leukemogenesis

Conditional deletion of *Pten* from $p16^{Ink4a}/p19^{Arf}$-deficient or *p53*-deficient backgrounds led to leukemogenesis (T-ALL, MPD, and/or histiocytic sarcoma) and to the death of mice within 14 days of pIpC treatment, much faster than after deletion of *Pten* from mice with wild-type tumor suppressors (data not shown). To avoid the rapid death of compound mutant mice and to compare the rates of leukemogenesis from limited numbers of mutant cells in a wild-type environment, we transplanted 1×10^6 unexcised donor ($CD45.2^+$) bone marrow cells from wild-type, $p16^{Ink4a}/p19^{Arf-/-}$, $Pten^{fl/fl}$;*Mx-1-Cre*$^+$, and $Pten^{fl/fl}$;*Mx-1-Cre*$^+$;$p16^{Ink4a}/p19^{Arf-/-}$ mice into irradiated wild-type recipient mice along with 500,000 recipient ($CD45.1^+$) bone marrow cells. Six weeks after transplantation, when the donor cells had stably engrafted, all recipients were treated with 7 injections of pIpC over 14 days to induce *Pten*-deletion. Recipients of wild-type and $p16^{Ink4a}/p19^{Arf-/-}$ bone marrow cells almost all survived for the duration of the experiment (165 days) with no signs of leukemogenesis (Fig. 5A). In contrast, recipients of $Pten^{fl/fl}$;*Mx-1-Cre*$^+$ cells died 49 to 162 days after ending pIpC treatment (Fig. 5A). Recipients of $Pten^{fl/fl}$;*Mx-1-Cre*$^+$;$p16^{Ink4a}/p19^{Arf-/-}$ cells died significantly ($p<0.02$) more quickly, 27 to 65 days after ending pIpC treatment (Fig. 5A). Histology confirmed that the mice had MPD and/or histiocytic sarcoma and/or T-ALL when they died. These data indicate that deficiency for $p16^{Ink4a}$ and $p19^{Arf}$ accelerates leukemogenesis after *Pten* deletion.

To better understand the relative contributions of $p16^{Ink4a}$ and $p19^{Arf}$ to the suppression of leukemogenesis after *Pten* deletion we performed the same experiment using mice that were compound mutants for *Pten* and $p16^{Ink4a}$ (Fig. 5C) or *Pten* and $p19^{Arf}$ (Fig. 5B). $p16^{Ink4a}$ deficiency did not significantly affect the rate at which mice died or the spectrum of hematopoietic neoplasms that arose after pIpC treatment (Fig. 5C). In contrast, $p19^{Arf}$ deficiency did significantly ($p<0.02$) increase the rate at which mice died after pIpC treatment (Fig. 5B). These results suggest that $p19^{Arf}$ suppresses leukemogenesis after *Pten* deletion, consistent with the increase in $p19^{Arf}$ expression in splenocytes after *Pten* deletion (Fig. 4A).

To assess the role of p53 we performed the same experiment using mice that were compound mutant for *Pten* and *p53* (Fig. 5D). Recipients of $Pten^{fl/fl}$;*Mx-1-Cre*$^+$;$p53^{-/-}$ cells died much more quickly after pIpC treatment as compared to recipients of $Pten^{fl/fl}$;*Mx-1-Cre*$^+$ cells ($p<0.0001$; Fig. 5D). All mice were confirmed to have MPD, AML, and/or T-ALL when they died (Fig. 5D). These results suggest that p53 suppresses leukemogenesis after *Pten* deletion.

$p16^{Ink4a}$ and p53 promote the depletion of HSCs after *Pten* deletion

To test whether the tumor suppressors that inhibit leukemogenesis after *Pten* deletion also act cell-autonomously within HSCs to promote their depletion, we transplanted 10 donor $CD150^+CD48^-CD41^-Lin^-c\text{-}kit^+Sca\text{-}1^+$ HSCs from mice with each of the genetic backgrounds depicted in Figure 6 into irradiated wild-type recipient mice, along with a radioprotective dose of 300,000 recipient bone marrow cells. The HSCs were isolated from the donor mice after they had been treated with 3 injections of pIpC over 6 days.

$p16^{Ink4a}$ deficiency prolonged the reconstituting capacity of *Pten*-deficient HSCs. Wild-type and $p16^{Ink4a}$-deficient HSCs gave long-term multilineage reconstitution by donor cells in all recipient mice (Fig. 6A). In contrast, *Pten*-deleted ($Pten^{fl/fl}$*Mx-1-Cre*$^+$) HSCs only gave transient multilineage reconstitution for 6 to 8 weeks after transplantation, consistent with our prior study (Yilmaz et al., 2006). Surprisingly, compound mutant $Pten^{fl/fl}$*Mx-1-Cre*$^+$$p16^{Ink4a-/-}$ HSCs gave significantly longer multilineage reconstitution than $Pten^{fl/fl}$*Mx-1-Cre*$^+$ HSCs (for 8 to 16 weeks, $p<0.002$; Fig. 6A) with significantly higher

donor cell reconstitution levels than the levels of reconstitution from *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$ HSCs (Fig. 6A). Sixteen weeks after transplantation 4 recipients of *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$*p16*$^{Ink4a-/-}$ HSCs remained alive and 2 of these mice remained multilineage reconstituted by donor cells. In a separate experiment, we confirmed that CD150^{+}CD48^{-}CD41^{-}Lin^{-}c-kit^{+}Sca-1^{+} donor HSCs (Fig. 6E) and CD150^{-}CD48^{-}CD41^{-}Lin^{-}c-kit^{+}Sca-1^{+} donor MPPs (Fig. 6G) could be recovered 8 weeks after transplantation from mice transplanted with 10 *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$*p16*$^{Ink4a-/-}$ HSCs but not with 10 *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$ HSCs. These data indicate that p16^{Ink4a} promotes the depletion of *Pten*-deficient HSCs.

Recipient mice that were transplanted with *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$*p16*$^{Ink4a-/-}$ HSCs did begin to die with MPD and/or T-ALL 12 to 16 weeks after transplantation whereas recipients of *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$ HSCs did not develop neoplasms (Fig. 6A). This difference partly reflects the fact that *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$ HSCs no longer had any donor cell chimerism by 12 weeks after transplantation and therefore were unable to develop donor cell leukemias (Fig. 6A). Therefore, *p16*^{Ink4a} deficiency increased the opportunity for leukemogenesis by prolonging the reconstituting capacity of *Pten*-deleted HSCs. These data might also reflect a limited role for p16^{Ink4a} in the suppression of leukemogenesis after *Pten* deletion.

p19^{Arf} deficiency did not rescue the depletion of *Pten*-deficient HSCs. Wild-type and *p19*^{Arf}-deficient HSCs gave long-term multilineage reconstitution by donor cells in all recipient mice (Fig. 6B). In contrast, *Pten*-deleted (*Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$) HSCs only gave transient multilineage reconstitution for 6 to 8 weeks after transplantation. Compound mutant *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$*p19*$^{Arf-/-}$ HSCs also gave transient multilineage reconstitution for only 4 to 8 weeks and at levels that were not significantly different from those observed from *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$ HSCs (Fig. 6B). This indicates that *p19*^{Arf} is not required for the depletion of HSCs after *Pten* deletion, consistent with our failure to detect *p19*^{Arf} expression in *Pten* deficient HSCs (Fig. 4C, D). Only a minority of recipients of *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$*p19*$^{Arf-/-}$ HSCs died with leukemia (beginning 8 weeks after transplantation), reflecting the transient reconstitution by these cells (Fig. 6B).

Consistent with the partial rescue of HSC depletion by *p16*^{Ink4a} deficiency, we also observed a partial rescue of HSC depletion by *p16*^{Ink4a}/*p19*^{Arf} deficiency. Wild-type and *p16*^{Ink4a}/*p19*^{Arf}-deficient HSCs gave long-term multilineage reconstitution by donor cells in almost all recipient mice (Fig. 6C). In contrast, *Pten*-deleted (*Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$) HSCs only gave transient multilineage reconstitution for 4 to 8 weeks after transplantation. Compound mutant *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$*p16*^{Ink4a}/*p19*$^{Arf-/-}$ HSCs gave significantly longer multilineage reconstitution than *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$ HSCs (for up to 14 weeks, $p<0.01$; Fig. 6C), with significantly higher donor cell reconstitution levels than from *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$ HSCs (Fig. 6C). These results are consistent with the data above in suggesting that p16^{Ink4a} depletes HSCs after *Pten* deletion. There were no statistically significant differences in the extent to which reconstitution was rescued by *p16*^{Ink4a} deficiency as compared to *p16*^{Ink4a}/*p19*^{Arf} deficiency, although *p16*^{Ink4a}/*p19*^{Arf} deletion was more leukemogenic (Fig. 6A, C).

p53 deficiency also prolonged the reconstituting capacity of *Pten*-deficient HSCs. Wild-type and *p53*-deficient HSCs gave long-term multilineage reconstitution by donor cells in all recipient mice for at least 16 weeks while *Pten*-deleted (*Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$) HSCs gave transient multilineage reconstitution for 4 to 6 weeks after transplantation (Fig. 6D). Compound mutant *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$*p53*$^{-/-}$ HSCs gave significantly longer multilineage reconstitution than *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$ HSCs (for up to 12 weeks, $p<0.002$; Fig. 6D), with significantly higher donor cell reconstitution levels than from *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$ HSCs (Fig. 6D). In a separate experiment, we confirmed that donor HSCs (Fig. 6F) and MPPs (Fig. 6H) could be recovered 8 weeks after transplantation of 10 *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$*p53*$^{-/-}$ HSCs but

not after transplantation of 10 $Pten^{fl/fl}Mx\text{-}1\text{-}Cre^{+}$ HSCs. p53 therefore contributes to the depletion of *Pten*-deficient HSCs in addition to suppressing leukemogenesis, consistent with its increased expression after *Pten* deletion in splenocytes and HSCs (Fig. 4).

The precise mechanisms by which $p16^{Ink4a}$ and p53 promote the depletion of HSCs after *Pten* deletion are uncertain. We did not detect any effect of *Pten* deletion or rapamycin treatment on the frequency of whole bone marrow cells or HSCs undergoing cell death (Fig. S7A) or expressing senescence-associated β-galactosidase (Fig. S7C–E). Indeed, we observed an increase in the frequency of dividing HSCs after *Pten* deletion (rather than a decrease as would be expected if senescence were occurring), and this increase was rescued by rapamycin treatment (Fig. S7B). These results suggest that increased $p16^{Ink4a}$ and p53 expression in dividing HSCs is incompatible with HSC maintenance, perhaps because these tumor suppressors promote maturation out of the stem cell pool.

Pten deficient leukemias have mutations that attenuate the tumor suppressor response

To test whether *Pten* deficient leukemias acquire secondary mutations that attenuate the tumor suppressor response we tested whether there was a selection pressure to inactive p53 in these leukemias. The tumor suppressors could be attenuated by many mechanisms that would silence or inactivate the tumor suppressors or other genes in the same pathways. Since it would be impossible to test all such mechanisms we circumvented this problem by studying leukemogenesis in a sensitized, *p53* heterozygous background. If there is selective pressure to inactivate p53 in *Pten* deficient leukemias, leukemias should arise more quickly from $p53^{+/-}$ mice as compared to $p53^{+/+}$ mice and we would expect the leukemias to delete the wild-type p53 allele. Young adult wild-type, $p53^{+/-}$, $Pten^{fl/fl};Mx\text{-}1\text{-}Cre^{+};p53^{+/+}$, and $Pten^{fl/fl};Mx\text{-}1\text{-}Cre^{+};p53^{+/-}$ mice were treated with pIpC and monitored. Wild-type and $p53^{+/-}$ mice all survived for the duration of the experiment (50 days) with no signs of leukemia, while $Pten^{fl/fl};Mx\text{-}1\text{-}Cre^{+};p53^{+/+}$ mice died 21 to 45 days after pIpC treatment with T-ALL and MPD (Fig. 7A). $Pten^{fl/fl};Mx\text{-}1\text{-}Cre^{+};p53^{+/-}$ mice died significantly (p=0.0008) more quickly, 8 to 39 days after ending pIpC treatment, again with T-ALL and MPD (Fig. 7A). Heterozygosity for p53 therefore accelerates leukemogenesis.

We next examined p53 expression in the thymus of mice that developed leukemias. In each case, the thymus was enlarged, completely effaced, and appeared to consist mainly of leukemic lymphoblasts. Whereas thymocytes from $Pten^{fl/fl};Mx\text{-}1\text{-}Cre^{+};p53^{+/+}$ mice showed robust expression of p53 (6 of 6 samples), thymyocytes from $Pten^{fl/fl};Mx\text{-}1\text{-}Cre^{+};p53^{+/-}$ mice showed little or no p53 expression (4 of 4 samples; Fig. 7B). PCR amplification of genomic DNA showed that thymic lymphoblasts from all four $Pten^{fl/fl};Mx\text{-}1\text{-}Cre^{+};p53^{+/-}$ mice had deleted the wild-type allele of *p53* (Fig. 7C). Thus, *Pten* deletion induces a strong selective pressure to inactive the tumor suppressor response in leukemogenic cells.

DISCUSSION

Pten deletion increased Akt, mTORC1, and S6 kinase activation in HSCs (Fig. 1B, D, E) but we could find no evidence for reduced FoxO1 or FoxO3a expression or cytoplasmic sequestration (Fig. 1B, F–H; Fig. S2). We observed a clear increase in ROS levels within thymocytes after *Pten* deletion but not in HSCs (Fig. 2). Consistent with this, NAC treatment attenuated the increase in ROS levels in thymocytes but did not rescue the changes in hematopoiesis, HSC frequency (Fig. 2), or HSC reconstituting capacity (Fig. 3A) after *Pten* deletion. This contrasted with results from *FoxO1/3/4*-deficient mice in which ROS levels clearly increased within HSCs and NAC treatment at least partially rescued HSC depletion (Tothova et al., 2007). *Pten* deletion and *FoxO1/3/4* deletion thus lead to the depletion of HSCs by different mechanisms. HSC depletion after *Pten* deletion is mediated

largely by mTOR activation with no evidence so far for an important contribution by oxidative stress.

Pten deletion induced a tumor suppressor response in hematopoietic cells, characterized by increased expression of $p19^{Arf}$ and p53 in splenocytes (Fig. 4A, B) and increased expression of $p16^{Ink4a}$ and p53 in HSCs (Fig. 4C–F). $p16^{Ink4a}/p19^{Arf}$ deficiency, $p19^{Arf}$ deficiency, or *p53* deficiency significantly accelerated leukemogenesis after *Pten* deletion (Fig. 5A, B, D). $p16^{Ink4a}$ deficiency did not significantly affect the rate of leukemogenesis after *Pten* deletion, though it did suppress the generation of histiocytic sarcomas (Fig. 5C). This suggests hematopoietic cells mainly rely upon the p53 pathway to suppress leukemogenesis after *Pten* deletion. This is consistent with results from mouse prostate in which *Pten* deletion induces p53-dependent senescence (Chen et al., 2005). Interestingly, this senescence response is $p19^{Arf}$-independent in prostate (Chen et al., 2009) but $p19^{Arf}$ did suppress leukemogenesis after *Pten* deletion, indicating tissue-specific functions for $p19^{Arf}$ in tumor suppression.

$p16^{Ink4a}$ deficiency, $p16^{Ink4a}/p19^{Arf}$ deficiency, or *p53* deficiency all significantly prolonged the ability of *Pten*-deficient HSCs to give multilineage reconstitution in irradiated mice (Fig. 6). $p19^{Arf}$ deficiency did not prolong the reconstituting capacity of *Pten*-deficient HSCs (Fig. 6B). Thus $p19^{Arf}$ is critical for the suppression of leukemogenesis but not for HSC depletion after *Pten* deletion. In contrast, $p16^{Ink4a}$ is critical for HSC depletion but plays a limited role suppressing leukemogenesis. One possible explanation for this distinction is that some leukemias may arise from cells other than HSCs and this process could be inhibited by $p19^{Arf}$ expression in those cells.

Our results thus indicate that *Pten* deletion induces an mTOR mediated tumor suppressor response in hematopoietic cells, suppressing leukemogenesis and depleting HSCs. This suggests that leukemias likely arise from rare clones of *Pten*-deficient hematopoietic cells that acquire secondary mutations that attenuate the tumor suppressor response. Consistent with this, we observed loss of *p53* heterozygosity in leukemias that arose from $Pten^{fl/fl}$;*Mx-1-*Cre^{+};$p53^{+/-}$ mice, indicating that *Pten* deletion imposes a strong selection against the tumor suppressor response (Fig. 7). It is also important to note that we do not know which hematopoietic cells are transformed after *Pten* deletion. Therefore, the tumor suppressors may act in HSCs themselves to suppress leukemogenesis or they may act in downstream cells.

Although $p16^{Ink4a}$ and $p19^{Arf}$ are both encoded at the *Cdkn2a* locus, they are regulated by different promoters, have no sequence homology, and different molecular functions (Sherr, 2001). The mechanisms by which *Pten* deletion or other oncogenic stimuli induce these tumor suppressor expression are not understood. While it is well established that oncogenic stresses such as c-Myc or E1A expression also activate the $p19^{Arf}$-p53 pathway (de Stanchina et al., 1998; Zindy et al., 1998), the mechanisms behind this activation remain unclear. The mechanisms behind $p16^{Ink4a}$ and p53 activation in response to Ras activation also remain unknown (Serrano et al., 1997).

The best-characterized consequences of p53 or $p16^{Ink4a}$ activation are senescence and apoptosis. However, these responses have only been characterized in certain non-stem cell populations, and it is possible that p53 or $p16^{Ink4a}$ activation may have other effects on stem cells. We did not detect any evidence that hematopoietic cells underwent senescence or cell death after *Pten* deletion (Fig. S7). However, HSCs are asynchronously depleted over a 4 to 8 week period after *Pten* deletion (Fig. 6). This raises the formal possibility that HSCs asynchronously undergo cell death or senescence over 4 to 8 weeks, such that very few HSCs express markers of cell death or senescence at any single time point, rendering it

undetectable. Nonetheless, the simplest interpretation of our data is that $p16^{Ink4a}$ and p53 expression cause HSCs to prematurely exit the stem cell pool, perhaps by maturing to transit amplifying MPPs. As this would occur asynchronously over time, the number of HSCs that prematurely exit the stem cell pool at any single time point would be imperceptibly small but the cumulative effect of premature maturation over a period of weeks would deplete HSCs.

Consistent with this model, deficiency for $p16^{Ink4a}$, $p19^{Arf}$, and *p53* dramatically expands the frequency of long-term multilineage reconstituting cells by conferring long-term self-renewal potential to MPPs which normally only give transient multilineage reconstitution (Akala et al., 2008; Kiel et al., 2008). These tumor suppressors thus play a physiological role promoting the transition from HSCs to MPPs and negatively regulating the self-renewal potential of multipotent cells. Increased expression of $p16^{Ink4a}$ and p53 in dividing HSCs after *Pten* deletion may accelerate the normal maturation of cells out of the HSC pool, leading to HSC depletion.

The ability to rescue the hematopoietic phenotypes in *Pten*-deficient mice with rapamycin suggests these phenotypes are driven by mTORC1 activation. However, our data indicate only that increased mTORC1 activation is required for HSC depletion and leukemogenesis, not that it is sufficient. This may explain why other genetic backgrounds that activate mTORC1, such as *Tsc1* deletion (Chen et al., 2008; Gan et al., 2008), do not necessarily lead to leukemogenesis. mTORC1-independent pathways downstream of Pten presumably also contribute to leukemogenesis. Since rapamycin can indirectly inhibit mTORC2 in addition to mTORC1 (Sarbassov et al., 2006) and mTORC2 is required for the development of prostate cancer after *Pten* deletion (Guertin et al., 2009), mTORC2 may mediate some of the effects of *Pten* deletion on HSCs and other hematopoietic cells.

The depletion of HSCs (and other hematopoietic progenitors) after *Pten* deletion may explain why few leukemias exhibit *Pten* deletion (Aggerholm et al., 2000; Chang et al., 2006; Sakai et al., 1998) other than T-ALL (Gutierrez et al., 2009). Rare clones of *Pten*-deficient hematopoietic stem/progenitor cells would be unlikely to have the opportunity to acquire secondary mutations before being depleted and therefore would be unlikely to progress to leukemia. Leukemias may be more likely to hyper-activate the PI-3kinase pathway by other types of mutations that are better tolerated by hematopoietic cells. Additional studies of the PI-3kinase pathway in stem cells will provide additional insights into stem cell regulation and the development of cancer.

MATERIALS AND METHODS

Mice

C57BL/Ka-Thy-1.1 (CD45.2) and C57BL/Ka-Thy-1.2 (CD45.1) mice were used in hematopoietic reconstitution experiments. $Mx\text{-}1\text{-}Cre^{+}$ mice (Kuhn et al., 1995), $Pten^{fl/fl}$ mice (Groszer et al., 2001), $Ink4a^{+/-}$ mice (Sharpless et al., 2001), $Arf^{+/-}$ mice (Kamijo et al., 1997), $Ink4a/Arf^{+/-}$ mice (Serrano et al., 1996), and $p53^{+/-}$ mice (Jacks et al., 1994) were backcrossed for at least ten generations onto a C57BL/Ka background. All mice were housed in the Unit for Laboratory Animal Medicine at the University of Michigan.

pIpC, rapamycin, and *N*-Acetyl-L-cysteine administration

Polyinosine-polycytidine (pIpC) and rapamycin were administered as previously described (Yilmaz et al., 2006). Briefly, pIpC from Sigma (St. Louis, MO) or from Amersham (Piscataway, NJ) was resuspended in Dulbecco's phosphate-buffered saline (D-PBS) at 2 mg/ml (for Sigma) or 50 μg/ml (for Amersham), and mice were injected intraperitoneally (IP) with 25 μg/gram (for Sigma) or 0.5 μg/gram (for Amersham) of body mass every other

day for 6–14 days. Rapamycin (LC Laboratories, Woburn, MA) was dissolved in absolute ethanol at 10 mg/ml and diluted in 5% Tween-80 (Sigma) and 5% PEG-400 (Hampton Research, Aliso Viejo, CA) before being administered daily by IP injection at a dose of 4 mg/kg. N-Acetyl-L-cysteine (NAC; Sigma) was administered daily by subcutaneous injection at 100 mg/kg (pH 7.0) starting one day after the final dose of pIpC.

Flow cytometry and HSC isolation

Bone marrow cells were obtained by crushing the long bones (tibias and femurs), pelvic bones, and vertebrae in a mortar and pestle with Hank's buffered salt solution without calcium or magnesium, supplemented with 2% heat-inactivated calf serum (GIBCO, Grand Island, NY; HBSS+). Cells were triturated and filtered through nylon screen (45 μm, Sefar America, Kansas City, MO) to obtain a single-cell suspension. For isolation of c-kit$^+$Flk2 Lin Sca-1$^+$CD48 cells, whole bone marrow cells were incubated with FITC-conjugated monoclonal antibodies to lineage markers including B220 (6B2), CD3 (KT31.1), CD4 (GK1.5), CD5 (53-7.3), CD8 (53-6.7), Gr-1 (8C5), Mac-1 (M1/70), and Ter119 (TER-119) in addition to APC-conjugated anti-Sca-1 (Ly6A/E; E13-6.7), biotin-conjugated anti-c-kit (2B8), and PE/Cy5-conjugated anti-Flk-2 (A2F10.1). Biotin-conjugated c-kit staining was visualized using streptavidin APC-Cy7.

For isolation of CD150$^+$CD48$^-$CD41$^-$Lin$^-$Sca-1$^+$c-kit$^+$ HSCs, bone marrow cells were incubated with PE-conjugated anti-CD150 (TC15-12F12.2; BioLegend, San Diego, CA), FITC-conjugated anti-CD48 (HM48-1; BioLegend), FITC-conjugated anti-CD41 (MWReg30; BD PharMingen, San Diego, CA), APC-conjugated anti-Sca-1 (Ly6A/E; E13-6.7), and biotin-conjugated anti-c-kit (2B8) antibody, in addition to antibodies against the following FITC-conjugated lineage markers: CD2 (RM2–5), B220 (6B2), CD3 (KT31.1), CD5 (53-7.3), CD8 (53-6.7), Gr-1 (8C5), and Ter119 (TER-119). Biotin-conjugated c-kit was visualized using streptavidin-conjugated APC-Cy7. HSCs were sometimes pre-enriched by selecting c-kit$^+$ cells using paramagnetic anti-biotin microbeads and autoMACS (Miltenyi Biotec, Auburn, CA). Dead cells were excluded using the viability dye 4′,6-diamidino-2-phenylindole (DAPI) (1 μg/ml).

To measure ROS levels, bone marrow cells were isolated and stained as above except that the HSC stain was modified to make the FITC channel available for DCFDA (2′-7′-dichlorofluorescein diacetate, Molecular Probes, Eugene, OR) staining. Antibodies for HSC isolation were as described above except for the following antibodies: PE/Cy5-conjugated anti-CD150 (TC15-12F12.2; BioLegend), PE-conjugated anti-CD48 (HM48-1; BioLegend), PE-conjugated anti-CD41 (MWReg30; BD PharMingen), and PE-conjugated antibodies against lineage markers. After antibody staining, thymus cells, whole bone marrow cells, or c-kit$^+$ enriched cells were incubated with 5 μM DCFDA for 15 min at 37°C followed by flow cytometry.

For details regarding immunofluorescence assays, western blotting and quantitative PCR see Supplementary Materials.

HIGHLIGHTS

- Oxidative stress is not a major driver of HSC depletion after *Pten* deletion
- mTOR activation after *Pten* deletion induces tumor suppressors that deplete HSCs
- p53 and p16^{Ink4a} suppress leukemia and promote HSC depletion after *Pten* deletion

- *Pten*-deficient leukemias acquire mutations that attenuate the tumor suppressors

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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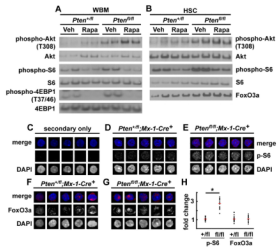

Figure 1. *Pten* deletion activated Akt and mTORC1 signaling in HSCs but FoxO3a was not inactivated

(A, B) *Pten* deletion increased phospho-Akt (T308), phospho-S6, and phospho-4EBP1 (T37/46) levels in whole bone marrow cells (A) as well as in c-kit^{+}Flk-2^{-}Lin^{-}Sca-1^{+}CD48^{-} HSCs (B) as expected. Rapamycin treatment tended to further increase phospho-Akt levels, but decreased phospho-S6, and phospho-4EBP1 (T37/46) levels, as expected. Quantification demonstrated that *Pten* deletion increased phospho-Akt levels by 2.6-fold and phospho-S6 levels by 1.5-fold by in HSCs. Rapamycin treatment further increased phospho-Akt levels by 1.7-fold in HSCs and decreased phospho-S6 levels by 40% in HSCs. Total protein levels of FoxO3a did not decrease with *Pten* deletion and were unaffected by rapamycin treatment (B). Each lane contained protein extracted from 40,000 sorted cells. (C–H) Staining of sorted CD150^{+}CD48^{-}CD41^{-}Lin^{-}c-kit^{+}Sca-1^{+} HSCs with secondary antibody alone (C), or primary and secondary antibody against phospho-S6 (D–E) or FoxO3a (F–G). Phospho-S6 staining was significantly elevated in *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$ HSCs as compared to *Pten*$^{+/fl}$*Mx-1-Cre*$^{+}$ control HSCs, as expected (D–E, H; *, $p<0.0001$ by Student's t-test), but the level and subcellular localization of FoxO3a staining did not differ between *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$ and control HSCs (F–H). We analyzed 10–30 HSCs from 1–2 mice/genotype in each of 3 independent experiments. In a similar assay, culture of HSCs in medium containing SCF and TPO did lead to decreased total FoxO3a levels and cytoplasmic localization (Fig. S3). See also Figures S1, S2, S3.

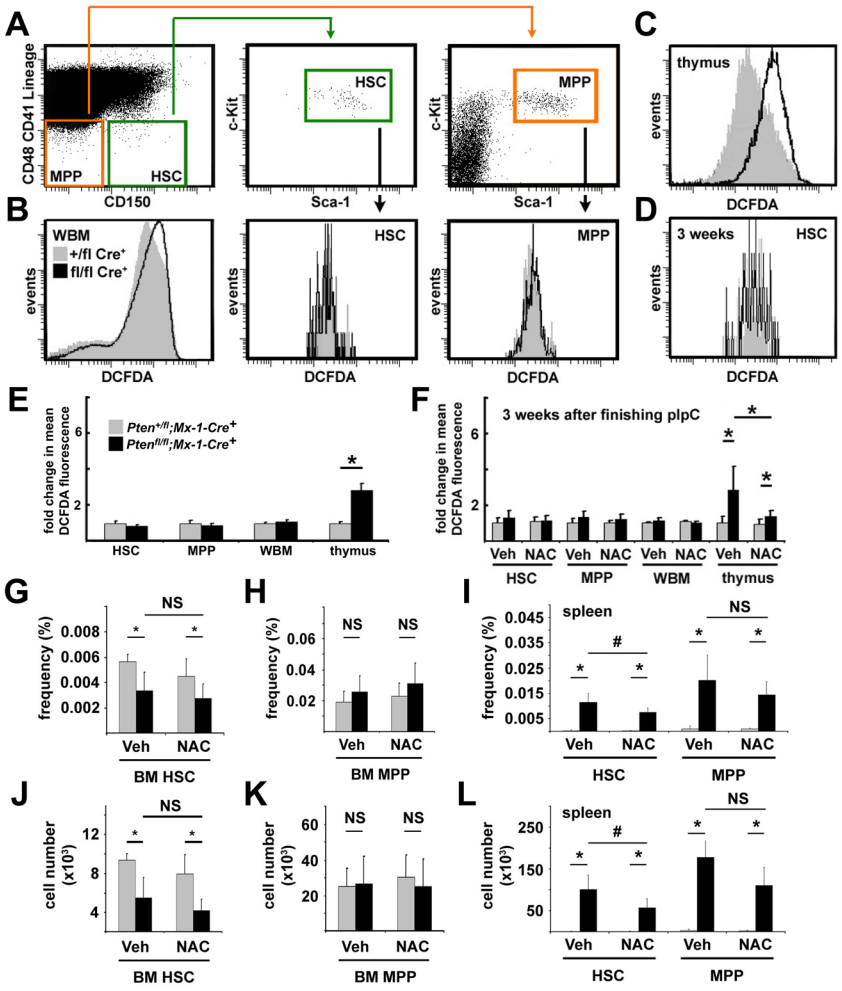

Figure 2. *Pten* deletion significantly increased ROS levels in thymocytes but not in HSCs, MPPs, or whole bone marrow cells and NAC treatment failed to rescue the depletion of HSCs

(A) Gating scheme used to assess intracellular ROS levels in $CD150^{+}CD48^{-}CD41^{-}Lin^{-}c\text{-}kit^{+}Sca\text{-}1^{+}$ HSCs and $CD150^{-}CD48^{-}CD41^{-}Lin^{-}c\text{-}kit^{+}Sca\text{-}1^{+}$ MPPs. 7 (B) or 21 (D) days after finishing pIpC treatment (7 doses of pIpC over 14 days), DCFDA staining of whole bone marrow cells, HSCs, and MPPs did not significantly differ between $Pten^{fl/fl}Mx\text{-}1\text{-}Cre^{+}$ and $Pten^{+/fl}Mx\text{-}1\text{-}Cre^{+}$ control mice. (C) In contrast, thymocytes from $Pten^{fl/fl}Mx\text{-}1\text{-}Cre^{+}$ mice did exhibit significantly greater DCFDA staining than thymocytes from $Pten^{+/fl}Mx\text{-}1\text{-}Cre^{+}$ controls. (E) Mean DCFDA fluorescence levels showed no evidence of increased ROS levels in HSCs, MPPs, or bone marrow cells, but a significant (*, $p<0.05$ by Student's t-test) increase in ROS levels within thymocytes. Similar experiments performed 21 days after finishing pIpC treatment yielded similar results (F). Daily subcutaneous injections of NAC after pIpC treatment did not significantly affect DCFDA staining of HSCs, MPPs, or bone marrow cells, but did significantly reduce DCFDA staining of thymocytes (F). The frequency (G) and absolute number (J) of $CD150^{+}CD48^{-}CD41^{-}Lin^{-}c\text{-}kit^{+}Sca\text{-}1^{+}$ HSCs in the bone marrow declined significantly after *Pten* deletion but were not affected by NAC. The frequency (H) and absolute number (K) of $CD150^{-}CD48^{-}CD41^{-}Lin^{-}c\text{-}kit^{+}Sca\text{-}1^{+}$ MPPs in the bone marrow were not affected by *Pten* deletion or NAC. The frequency (I) and absolute number (L) of HSCs and MPPs in the spleen significantly increased after *Pten* deletion. The increase in HSCs was slightly but significantly (#, $p<0.05$) attenuated by NAC treatment but the increase in MPPs was not significantly affected (I, L). Data (mean

±standard deviation) are from 4 independent experiments with 1–2 mice/genotype/treatment. See also Figure S4.

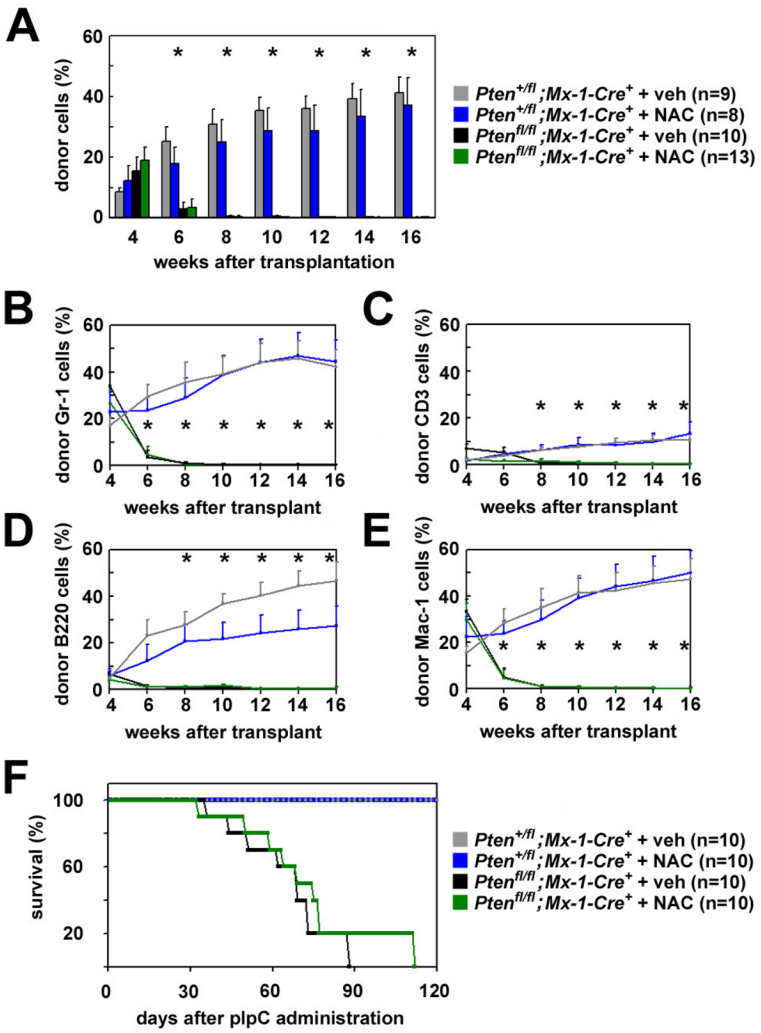

Figure 3. NAC treatment did not restore the reconstituting capacity of HSCs or block leukemogenesis after *Pten*-deletion

(A–E) After pIpC treatment, 10 donor $CD150^{+}CD48^{-}CD41^{-}Lin^{-}c\text{-}kit^{+}Sca\text{-}1^{+}$ HSCs were transplanted into lethally irradiated recipients along with 300,000 recipient bone marrow cells, and recipients were maintained on daily injections of NAC or vehicle beginning the day after transplantation. Control (*Pten*$^{+/fl}$*Mx-1-Cre*$^{+}$) HSCs gave high levels of long-term multilineage reconstitution by donor Gr-1+ myeloid (B), CD3+ T (C), B220+ B (D), and Mac-1+ myeloid cells (E) in all recipients, irrespective of NAC treatment. *Pten*-deleted (*Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$) HSCs gave transient multilineage reconstitution in all recipients, and significantly (*, $p<0.05$ by Student's t-test) lower levels of donor reconstitution in all lineages (BE), irrespective of NAC treatment. NAC treatment did not significantly affect reconstitution levels from either *Pten*-deleted or control HSCs. Data represent mean±SEM from 3 independent experiments. (F) In 2 independent experiments, 1×10^{6} unexcised donor cells from *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$ or *Pten*$^{+/fl}$*Mx-1-Cre*$^{+}$ mice were transplanted into irradiated recipient mice. Six weeks later *Pten* was deleted by pIpC treatment, then recipients were given daily injections of NAC or vehicle. NAC treatment did not prolong the survival of mice or delay the onset of leukemia after *Pten* deletion. None of the recipients of control cells developed neoplasms but all mice with *Pten*-deficient cells had MPD and/or T-ALL when they died, irrespective of NAC treatment. See also Figure S5.

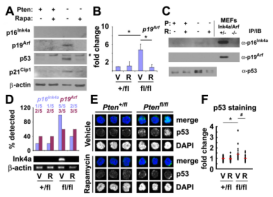

Figure 4. *Pten* deletion increased p19^{Arf}, p21^{Cip1}, and p53 expression in splenocytes, and p16^{Ink4a} and p53 in HSCs, and rapamycin attenuated these increases

(A) Levels of p16^{Ink4a}, p19^{Arf}, p21^{Cip1}, and p53 were assessed by Western blot in unfractionated splenocytes from *Pten$^{fl/fl}$Mx-1-Cre^{+}* mice and *Pten$^{+/fl}$Mx-1-Cre^{+}* controls 14 days after pIpC treatment. Mice were given daily injections of rapamycin or vehicle after pIpC treatment ended. Analysis of *p53*-deficient MEFs (data not shown) indicated that the upper band (#) was not specific for p53 but the lower band was. This blot is representative of 3 independent experiments. (B) We also observed increased *p19^{Arf}* transcript levels by qPCR in splenocytes after *Pten* deletion and this effect was attenuated by rapamycin treatment (mean±SD from 3 independent experiments). (C) 4 weeks after pIpC treatment ended, 2×10^6 Lin$^-$c-kit$^+$ stem/progenitor cells were sorted from control and *Pten*-deleted mice and cell lysates were immunoprecipitated using antibodies against p16^{Ink4a}, p19^{Arf}, and p53 before Western blotting. p16^{Ink4a} and p53 levels increased in *Pten*-deleted cells, but we detected no increase in p19^{Arf}. MEFs that were deficient or heterozygous for *p16^{Ink4a}/p19^{Arf}* were used as negative and positive controls. (D) *p16^{Ink4a}* transcript could always be amplified from *Pten* deficient CD150$^+$CD48$^-$CD41$^-$Lin$^-$c-kit$^+$Sca-1$^+$ HSCs (5 of 5 samples) but usually not from control (1 of 5) or rapamycin-treated *Pten* deficient samples (2 of 5) 4 weeks after *Pten* deletion. *p19^{Arf}* transcripts could only be amplified from about half of the samples, irrespective of *Pten* deletion or rapamycin treatment (data are from 5 independent experiments). (E, F) 4 weeks after pIpC treatment ended, CD150$^+$CD48$^-$CD41$^-$Lin$^-$c-kit$^+$Sca-1$^+$ HSCs from *Pten*-deleted mice exhibited higher levels of immunofluorescence for p53 than control HSCs or *Pten* deleted HSCs treated with rapamycin. (F) The average staining intensity for p53 increased 1.4-fold (*, $p<0.008$ by Student's t-test) in *Pten*-deleted HSCs as compared to control HSCs. Rapamycin treatment rescued this effect (#, $p<0.003$ by Student's t-test; data are from 30 HSCs per group compiled from 2 independent experiments). None of the mice studied in this figure showed any signs of hematopoietic neoplasms. See also Figure S6.

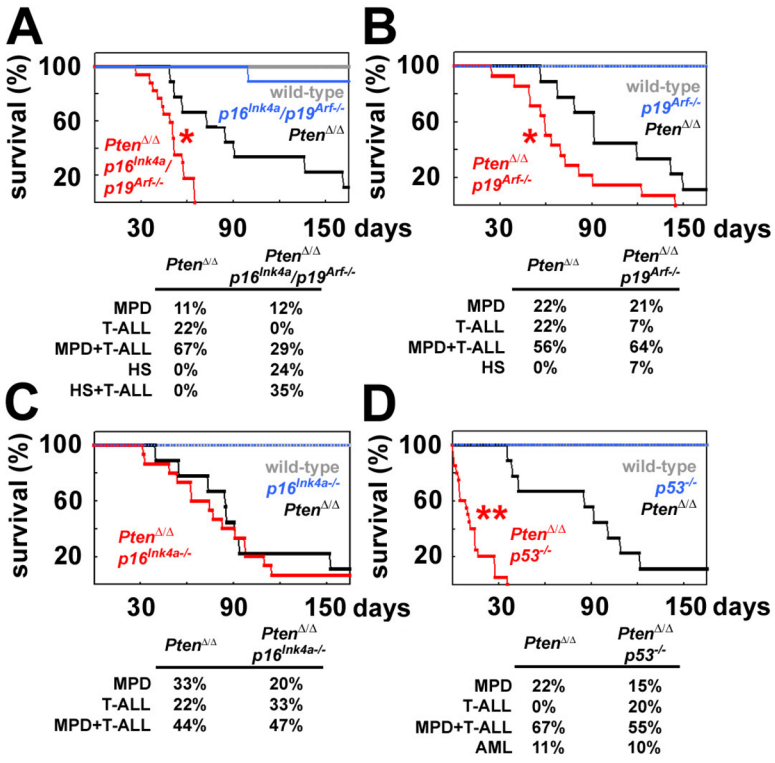

Figure 5. Deficiency for *p19^Arf^* or *p53*, but not *p16^Ink4a^*, accelerated leukemogenesis after *Pten*-deletion

1×10^6 donor bone marrow cells from mice with the indicated genotypes were transplanted into irradiated recipient mice along with 500,000 recipient bone marrow cells. Six weeks after transplantation, all recipients were treated with pIpC and their survival was monitored over time (up to 165 days after pIpC treatment ended). (A) Recipients of *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$*p16*^{Ink4a}/*p19*$^{Arf-/-}$ cells (displayed as *Pten*$^{\Delta/\Delta}$*p16*^{Ink4a}/*p19*$^{Arf-/-}$) exhibited significantly (*, p<0.02 by Student's t-test) accelerated death as compared to recipients of *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$ cells (*Pten*$^{\Delta/\Delta}$). Mice were sacrificed when moribund and their hematopoietic tissues analyzed. The neoplasms observed in each mouse at the time of sacrifice included myeloproliferative disease (MPD), T-ALL, MPD+T-ALL, histiocytic sarcoma (HS), and HS+T-ALL. (B) Recipients of *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$*p19*$^{Arf-/-}$ cells (*Pten*$^{\Delta/\Delta}$*p19*$^{Arf-/-}$) exhibited significantly (*, p<0.02 by log-rank test) accelerated death from leukemogenesis as compared to recipients of *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$ cells (*Pten*$^{\Delta/\Delta}$). (C) Recipients of *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$*p16*^{Ink4a} (*Pten*$^{\Delta/\Delta}$*p16*^{Ink4a}) cells died at a similar rate and with similar neoplasms as recipients of *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$ cells (*Pten*$^{\Delta/\Delta}$). (D) Recipients of *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$*p53*$^{-/-}$ cells (*Pten*$^{\Delta/\Delta}$*p53*$^{-/-}$) exhibited significantly (**, p<0.0001 by log-rank test) accelerated death from leukemogenesis as compared to recipients of *Pten*$^{fl/fl}$*Mx-1-Cre*$^{+}$ cells (*Pten*$^{\Delta/\Delta}$). Data are from 3 independent experiments with a total of 9 mice/genotype except for compound mutant mice, which had 14–20 mice/genotype.

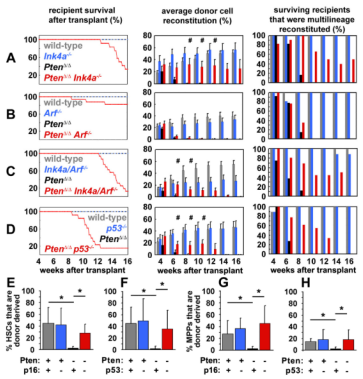

Figure 6. Deficiency for *p16*^{Ink4a} or *p53* prolonged the reconstituting capacity of *Pten*-deficient HSCs

(A) 10 CD150^{+}CD48^{-}CD41^{-}Lin^{-}c-kit^{+}Sca-1^{+} cells were sorted from mice with each of the indicated genotypes after pIpC treatment and co-injected with 300,000 recipient bone marrow cells into irradiated recipient mice. The survival, donor cell reconstitution levels (mean±SEM), and percentage of surviving recipients with multilineage reconstitution by donor cells were monitored for 16 weeks after transplantation. In all experiments, recipients of wild-type cells (A–D) and recipients of *p16*^{Ink4a}-deficient cells (A), *p19*^{Arf}-deficient cells (B), *p16*^{Ink4a}/*p19*^{Arf}-deficient cells (C) or *p53*-deficient cells (D) survived for the duration of the experiment and showed high levels of long-term multilineage reconstitution by donor cells. In contrast, recipients of *Pten*-deficient cells showed only transient multilineage reconstitution for 4 to 8 weeks in each experiment (A–D). HSCs that were compound mutant for *Pten* in addition to *p16*^{Ink4a} (A), *p16*^{Ink4a}/*p19*^{Arf} (C) or *p53* (D) gave significantly (#, $p<0.05$ by Student's t-test) higher levels of donor cell reconstitution and multilineage reconstitution for a significantly longer period of time as compared to HSCs that were deficient only for *Pten*. The degree of donor cell reconstitution provided by *Pten*$^{fl/fl}$;*Mx-1-Cre*$^{+}$;*p16*^{Ink4a} cells (A) did not significantly differ from *Pten*$^{fl/fl}$;*Mx-1-Cre*$^{+}$;*p16*^{Ink4a}/*p19*^{Arf} cells (C). *p19*^{Arf} deficiency did not significantly affect the duration or level of reconstitution by *Pten*-deficient HSCs (B). Compound mutant mice that died had MPD, T-ALL, and/or histiocytic sarcoma at the time of death. All data are from 3 independent experiments with a total of 7–17 recipients per treatment. (E–H) Mice were transplanted with mutant HSCs as described above then sacrificed 8 weeks later to assess the frequency of donor CD150^{+}CD48^{-}CD41^{-}Lin^{-}c-kit^{+}Sca-1^{+} HSCs and CD150^{-}CD48^{-}CD41^{-}Lin^{-}c-kit^{+}Sca-1^{+} MPPs. Donor HSCs and MPPs were not detectable by this time point in the absence of *Pten* but depletion was rescued by either *p16*^{Ink4a} deficiency (E,G) or *p53* deficiency (F,H; *, $p<0.05$ by Student's t-test). These data are from 3 independent experiments with a total of 3–8 recipients per treatment. Consistent with Figure 6, transplantation of higher doses of *Pten*-deficient CD150^{+}CD48^{-}CD41^{-}Lin^{-}c-kit^{+}Sca-1^{+} cells into irradiated wild-type mice led to the development of leukemia in a higher proportion of the recipient mice (Table S1). See also Figure S7 and Table S1.

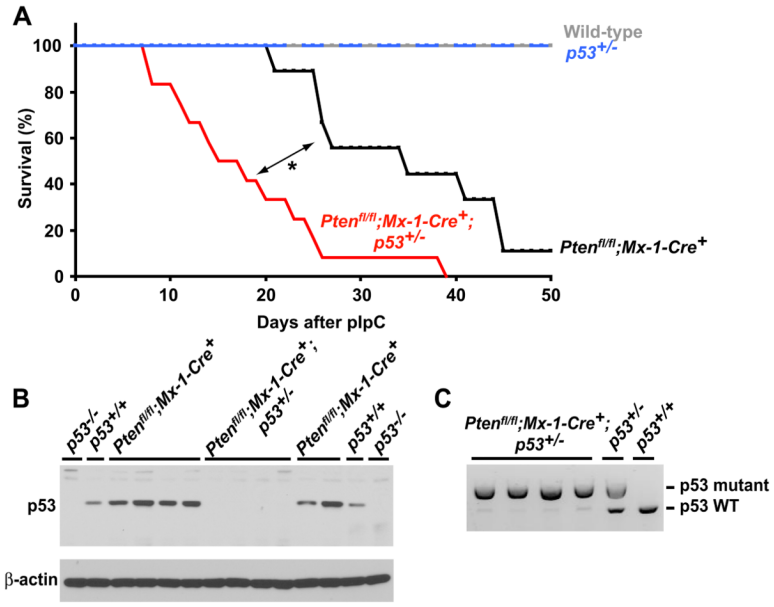

Figure 7. Some *Pten*-deficient leukemias inactivate p53

(A) Wild-type, $p53^{+/-}$, $Pten^{fl/fl}$;*Mx-1-Cre*$^{+}$;$p53^{+/+}$, and $Pten^{fl/fl}$;*Mx-1-Cre*$^{+}$;$p53^{+/-}$ mice 5 to 6 weeks of age were treated with three doses of pIpC then monitored. $Pten^{fl/fl}$;*Mx-1-Cre*$^{+}$;$p53^{+/-}$ mice died significantly more quickly than $Pten^{fl/fl}$;*Mx-1-Cre*$^{+}$;$p53^{+/+}$ mice (p=0.0008 by log-rank test). (B) Western blotting of thymocytes from $Pten^{fl/fl}$;*Mx-1-Cre*$^{+}$;$p53^{+/+}$ and $Pten^{fl/fl}$;*Mx-1-Cre*$^{+}$;$p53^{+/-}$ mice with T-ALL showed strongly diminished expression of p53 in the $Pten^{fl/fl}$;*Mx-1-Cre*$^{+}$;$p53^{+/-}$ cells. (C) Genomic PCR analysis of thymocytes from $Pten^{fl/fl}$;*Mx-1-Cre*$^{+}$;$p53^{+/-}$ mice showed a significantly diminished band corresponding to the wild-type *p53* allele suggesting loss of heterozygosity. The weak wild-type p53 band may reflect residual normal thymocytes or thymic stromal cells. 9–12 mice/genotype were used in this experiment.